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(54) Title: DETECTION OF BIOLOGICAL MATERIAL

(57) Abstract

A method for detecting the presence of any of the materials C_1, C_2, enzyme E_1 (having the substrates C_1 and S_1) and enzyme E_2 (having the substrates C_2 and S_2) that undergo the following reactions (I) and (II). This method comprises adding the other materials sufficient for the reactions to proceed, and observing the loss of S_1 or S_2 or the formation of P. The formation of P increases exponentially, and that can be used to give a colour reaction quickly and simply, with a suitable P-linked reaction, in the presence of microorganisms (C_1 being ATP).

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DETECTION OF BIOLOGICAL MATERIAL

Field of the Invention

This invention relates to a method for the detection of biological material, and to materials for use in such a method.

Background of the Invention

Many techniques are available for the detection of biological materials, for instance by the production of a colour. For example, materials may be added that form hydrogen peroxide or NADH in the presence of the target substance, and these may be caused to produce a coloured compound by further reactions. Alternatively, the target substance may produce colour more directly; for instance, many hydrolytic enzymes can act on artificial substrates to give a coloured product.

A difficulty arises, however, in connection with the detection of very small amounts of the target substance, such that the amount of colour produced may be too small to analyse without complicated apparatus, and certainly not by the naked eye. Some amplification mechanisms have been described, such as cyclic reactions for the measurement of low levels of NAD(H) or NADP(H); see EP-A-0060123. This is an example of a cycle giving linear amplification of the target substances, i.e. their concentrations increase in proportion with reaction time, and therefore a long period is required for sensitive measurements.

GB-A-2055200 discloses the same and other linear amplification cycles. It also discloses a cycle wherein adenylate kinase catalyses the reaction of AMP and ATP to form ADP which is then re-phosphorylated to form ATP by the action of pyruvate kinase. It is stated that, in each cycle, one extra molecule of ATP is produced. The reaction is used to amplify ATP for the purposes of bioluminescence. The procedure for ATP amplification involves adding the other materials sufficient for the reactions to proceed, stopping the cycling reaction, and measuring accumulated ATP using a bioluminescence assay and a luminometer.

Chittock et al, Biochemical Society Transactions (1991) 19:160S, also disclose that the light intensity of the known bioluminescence reaction may be increased by recycling ATP in a system containing myokinase (adenylate kinase), pyruvate kinase, phosphoenolpyruvate and AMP. The authors consider the fate of two molecules of ATP present initially in the sample. One molecule is consumed by the luciferase reaction and is converted to AMP. It can then undergo a reaction with the other molecule of ATP, in a reaction catalysed by myokinase (adenylate kinase), to form two molecules of ADP. These are subsequently re-phosphorylated by reaction with phosphoenolpyruvate, catalysed by pyruvate kinase, to regenerate the original two molecules of ATP. The added AMP is described as a "trap" for ATP which helps to get the recycling reaction underway.

It is not clear from the reaction scheme proposed by Chittock et al why the proposed ATP recycling mechanism should cause the observed increase in peak light output of the luciferase reaction, since no net increase in ATP concentration would occur. Nevertheless, the authors describe the phenomenon as amplification of ATP.

Summary of the Invention

It has now been appreciated that such a system can be used, not simply for the regeneration of ATP, but for the production of a colour or other easily-detectable signal which can be used to quantify very low levels of ATP and ADP. The signal may be visible, avoiding the previous requirement for additional apparatus such as a luminometer.

More generally, according to the present invention, coupled reactions of the type shown below in Fig. 1 are used to detect the presence of either or both of the interconvertible compounds C_1 and C_2 or of the enzymes E_1 and E_2, by the provision of the other components of the cycle necessary for the reactions to proceed, including the respective enzyme substrates S_1 and S_2, and observation of the loss of a substrate (S_1 or S_2) or, preferably, the

formation of a product (P). By comparison with the procedure disclosed in GB-A-2055200, the exponential amplification is used for the observation of an extra-cyclic component.

Observation may be direct or by coupling the reactions to a system that gives a detectable derivative (or derivative reaction) and which may be known per se. Advantages associated with the present invention are that a yes/no result can be obtained, that the reaction is quick, and that it can be used to detect microorganisms.

Description of the Drawings

The invention will be described by way of example only with reference to the accompanying drawings, in which:

Figures 1 to 5 are each schemes of reactions, more or less general, that may be utilised in the present invention; and

Fig. 6 is a graph representing results obtained by the invention.

Abbreviations used in Figs. 4 and 5, and elsewhere, have the following meanings:

AMP: Adenosine 5′-monophosphate
ADP: Adenosine 5′-diphosphate
ATP: Adenoise 5′-triphosphate
G6P: D-Glucose 6-phosphate
G: D-Glucose
AAP: 4-Aminoantipyrine
DMA: N,N-dimethylaniline
G_{ox}: D-Glucono-1,5-lactone
AK: Adenylate kinase
GK: Glucokinase
GO: Glucose oxidase
HRP: Horseradish peroxidase
G1P: D-Glucose 1-phosphate
G1,6DP: D-Glucose 1,6-bisphosphate
3PGP: 3-Phospho-D-glyceroyl phosphate
3PG: 3-Phospho-D-glycerate
3PHP: 3-Phosphohydroxypyruvate

GPPDM: Glucose-1-phosphate phosphodismutase
GBPS: Glucose-1,6-bisphosphate synthase
PGDH: Phosphoglycerate dehydrogenase
DPA: Diaphorase
Col: Colour
INV: INT-violet
Fzn: Formazan.

Description of the Invention

The coupled reactions on which the present invention are based are illustrated generally by Fig. 1. By way of illustration only, a description of how the cycle can be used to detect a pair of interconvertible compounds C_1 and C_2 is given below. A similar mechanism would apply for the detection of one or both of the enzymes E_1 and E_2.

The reaction catalysed by enzyme E_1 is a "comproportionation" reaction, in which one molecule of the first target compound C_1 reacts with one molecule of added substrate S_1 to form two molecules of the second target compound C_2. By the action of enzyme E_2 and its co-substrate S_2, these two molecules of C_2 are now converted to two molecules of the first target compound C_1 which are available as substrates for the enzyme E_1. Thus one molecule of the first target compound has been converted to two: another turn of the cycle will produce four, yet another eight, etc.

More specifically, the reactions that take place in cycles 1, 2 and 3 are as follows:

1) $C_1 + S_1 \rightarrow 2C_2$ and $2C_2 + 2S_2 \rightarrow 2C_1 + 2P$
2) $2C_1 + 2S_1 \rightarrow 4C_2$ and $4C_2 + 4S_2 \rightarrow 4C_1 + 4P$
3) $4C_1 + 4S_1 \rightarrow 8C_2$ and $8C_2 + 8S_2 \rightarrow 8C_1 + 8P$

In this way, an exponential amplification of a target compound occurs. Molecules of the second target compound present initially will be amplified in a similar way.

With reference to Fig. 1, there is a choice of methods by which the progress of the cycle may be followed. As the cycle proceeds, the concentrations of S_1 and S_2 will decrease, whilst the concentrations of C_1, C_2 and P will

increase. Any of these changes in concentration of an extra-cyclic material might be suitable for linking to, by way of example, the production or loss of a coloured compound. If it is not possible to cause a colour change to occur in the same reaction system as the amplification cycle, then samples could be removed for separate analysis; removal of P may facilitate the cyclic reactions.

Figs. 2 and 3 illustrate more specific examples of the invention, in which interconversion of the two target compounds involves transfer of a chemical group. More generally, analogous mechanisms exist involving, for instance, transfer of one or more electrons between redox-active substances.

X-Y-Y and X-Y in Fig. 2 might be compounds such as ATP and ADP respectively, which differ only by one phosphate group. Enzyme E_1 in this case would be adenylate kinase (E.C. 2.7.4.3) which catalyses the "comproportionation" reaction in which one molecule of the first target substance X-Y-Y (e.g. ATP) reacts with one molecule of added substrate X (e.g. AMP) to form two molecules of the second target substance X-Y (e.g. ADP). Enzyme E_2 in this example could be a kinase which, together with added phosphorylated co-substrate Z-Y, would phosphorylate ADP to ATP.

Fig. 3 shows an alternative cycle in which the flow of the chemical group Y is, essentially, in the opposite direction. In this case, the two target compounds are X and X-Y.

Fig. 4 illustrates the linking of the enzymatic cycle of Fig. 2 to colour production, for the case of ADP/ATP amplification. Enzyme E_1 is adenylate kinase and enzyme E_2 is glucokinase, which produces D-glucose and ATP from D-glucose-6-phosphate and ADP. The increase in concentration of glucose is shown to be monitored by the linked reactions of glucose oxidase (E.C. 1.1.3.4) and horseradish peroxidase (E.C. 1.11.1.7) together with suitable chromogenic substrates. Many alternative kinase

enzymes to serve as E_2 are available together with their specific co-substrate: for instance, pyruvate kinase (E.C. 2.7.1.40) with phosphoenolpyruvate, acetate kinase (E.C. 2.7.2.1) with acetyl phosphate, NADH kinase (E.C. 2.7.1.86) with NADPH, fructokinase (E.C. 2.7.1.4) with D-fructose-6-phosphate, or glycerol kinase (E.C. 2.7.1.30) with glycerol-3-phosphate. In fact, many enzymes from the group 2.7 in the IUB Recommendations on Enzyme Nomenclature (1984, Academic Press) might be used in such a scheme. In each case, the product may be detected by a specific enzyme reaction, a series of enzyme reactions, or a chemical reaction, producing or destroying a colour.

If desired, contaminating levels of ATP and ADP in the amplifying reagent can be lowered by an initial incubation of the components of the reagent with an enzyme such as potato apyrase (E.C. 3.6.1.5) which hydrolyses both ATP and ADP to AMP. Provided it is at a low concentration, the presence of apyrase will not interfere with the amplification cycle.

Thus a reagent for the detection of ATP and/or ADP might comprise AMP, adenylate kinase, glucokinase, D-glucose-6-phosphate, glucose oxidase, apyrase, horseradish peroxidase and one or more chromogenic peroxidase substrates in a suitable buffer system containing magnesium ions. Since the cycle is essentially exponential, a low amount of ADP or ATP (perhaps a single molecule) present in the complete reagent will rapidly be multiplied until one of the substrates is exhausted. If desired, the reagent may be prepared as two or more separate solutions which are mixed together at approximately the same time as a sample for assay is added.

Figs. 2 and 4 show that one of the interconvertible compounds which can be detected is ATP. The present invention is thus particularly suitable as a ready test for the presence of microorganisms. A liquid sample containing a very low concentration of microorganisms can be tested by, for example, presentation on a carrier, e.g. a multi-

well plate, and adding the necessary reagents for the reaction to proceed. The process described in WO-A-9319199 may be used to retain microorganisms in wells, even when at very low concentration in a liquid sample. The present invention can detect the microorganisms in each well, giving a result that is available to the naked eye, within a reasonable period of time.

Alternatives to the ATP/ADP amplification cycle exist. The requirements are (i) an enzyme catalysing a comproportionation reaction, such that the presence of one molecule of the first target substance causes the formation of two molecules of the second target substance, and (ii) an enzyme which catalyses the formation of the first target substance from the second. An example is shown in Fig. 5. Here, E_1 is glucose-1-phosphate phosphodismutase (E.C. 2.7.1.41), E_2 is glucose-1,6-bisphosphate synthase (E.C. 2.7.1.106), and the target substances are D-glucose-1,6-bisphosphate and D-glucose-1-phosphate. Progress of the amplification cycle may be followed by using the 3-phospho-D-glycerate produced by E_2 as a substrate for the enzyme phosphoglycerate dehydrogenase (E.C. 1.1.1.95), producing NADH which can be used to form a colour by chemical or enzymatic means: for instance, the reaction with INT-violet catalysed by the enzyme diaphorase (E.C. 1.8.1.4).

The following Examples illustrate the invention.

Example 1

Two reagent pre-mixes, A and B, were prepared with the following compositions (MES = 2-(N-morpholino)ethane-sulphonic acid):

Pre-mix A

D-glucose 6-phosphate	2.67 mM
Adenosine 5'-monophosphate (AMP)	1.33 mM
Adenylate kinase	0.27 U/ml
Bovine serum albumin	0.2 mg/ml
EDTA	0.67 mM
4-Aminoantipyrine	1.33 mM

N,N-dimethylaniline	3.33 mM
Magnesium chloride	20 mM
Potassium chloride	40 mM
Potato apyrase	0.04 U/ml
MES pH 6.0	100 mM

Pre-mix B

Glucose oxidase	320 U/ml
Glucokinase	80 U/ml
Horseradish peroxidase	32 U/ml
Bovine serum albumin	0.2 mg/ml
Magnesium chloride	20 mM
Potassium chloride	40 mM
Potato apyrase	0.04 U/ml
MES pH 6.0	100 mM

The pre-mixes were incubated separately at 25°C for 3 hours, to destroy contaminating ATP and ADP. Then, in each of three cuvettes, 0.225 ml of Pre-mix A was mixed with 0.075 ml of Pre-mix B, and 0.3 ml of a sample was immediately added. The samples comprised water (as a blank), 20 pM ATP and 200 pM ATP. The absorbance at 555 nm in each cuvette was followed in a spectrophotometer during incubation for 200 minutes at 25°C.

Plots of absorbance (A) with respect to time (T) are presented in Figure 6. It should be noted that, after a reaction time of approximately 150 minutes, the three samples could easily be distinguished by eye. The more ATP that was added, the sooner a violet colour appeared in the solution.

Example 2

A similar reagent composition was used for the rapid enumeration of E. coli using the most probable number technique. This approach is based on dilution of the culture and its even distribution into a number of compartments (in this case 24 per sample), with dilutions chosen such that each set is likely to include compartments with either no bacteria or a small number. Addition of growth medium then allows multiplication of the bacteria to

levels that can be detected, and the number of compartments positive for growth gives an estimate of the sample bacterial population. This general technique is described in WO-A-9319199.

A microtitre plate with 0.2 μm membrane at the bottom of each well was set up as follows, using dilutions of an overnight culture of E. coli in 0.9% (w/v) NaCl containing 5 mM magnesium chloride:

Columns 1-6: 0.04 ml diluent
Columns 7-9: 0.04 ml E. coli, 10^{-8} dilution
Columns 10-12: 0.04 ml E. coli, 2.5×10^{-8} dilution.

Vacuum was applied to remove the liquid and to collect the bacteria on the membrane filters. 0.1 ml of sterile yeast extract-peptone broth was then added to each well, and the microplate was covered and incubated at 37°C for 8.5 hours.

At the end of the incubation period, the broth was removed by vacuum, and the wells were each washed with 0.25 ml of sterile diluent in order to remove non-microbial ADP and ATP (which are present in broth). After vacuuming again, 0.025 ml of 0.22% (w/v) chlorohexidine digluconate (a cationic detergent used for lysing bacteria) was added to each well, followed by 0.025 ml of diluent. For columns 1-6, the diluent was spiked with various concentrations of ATP between 0 and 6×10^{-9}M.

The two Pre-mixes, similar to Example 1 but with concentrations of the constituents adjusted to take account of the different sample volume in this experiment, were rapidly mixed together and 0.1 ml volumes were immediately added to each well. The plate was placed in a CERES 900 HDi microplate reader (Bio-Tek) incubated at 30°C, and the absorbances were measured at 550 nm for up to three hours.

Clear results were obtained after 70 minutes. At this point, in columns 1-6, only the highest ATP standard (10^{-9}M in the final reaction mixture) had developed a measurable amount of colour. In columns 7-12, the absorbance in the wells was either very close to the starting value (i.e. a

negative) or was clearly detectable (a positive, showing that at least one bacterium had been present initially). Columns 7-9 had 15 positives, which equates to a most probable number of 24.5 per ml (95% confidence range 13-42), and columns 10-12 had 21 positives, giving a most probable number of 52 per ml (95% confidence range 28-91). Conventional plate counts on yeast extract-peptone agar of the initial E. coli culture gave estimates of 29 and 71 cfu/ml respectively for the two dilutions.

The procedure therefore took less than 10 hours to enumerate the E. coli samples. The degree of colour development was sufficient to allow reading of the plate by eye.

CLAIMS

1. A method for detecting the presence of any of the materials C_1, C_2, enzyme E_1 (having the substrates C_1 and S_1) and enzyme E_2 (having the substrates C_2 and S_2) that undergo the following reactions

$$C_1 + S_1 \xrightarrow{E_1} 2\ C_2$$

$$C_2 + S_2 \xrightarrow{E_2} C_1 + P$$

which comprises adding the other materials sufficient for the reactions to proceed, and observing the loss of S_1 or S_2 or the formation of P.

2. A method according to claim 1, for detecting the presence of any of the materials X-Y, X-Y-Y, enzyme e_1 and enzyme e_2 that undergo the following reactions

$$X + X\text{-}Y\text{-}Y \xrightarrow{e_1} 2\ X\text{-}Y$$

$$X\text{-}Y + Z\text{-}Y \xrightarrow{e_2} X\text{-}Y\text{-}Y + Z$$

which comprises adding the other materials sufficient for the reactions to proceed, and observing the loss of X or Z-Y or the formation of Z.

3. A method according to claim 1, for detecting the presence of any of the materials X, X-Y, enzyme e_1 and enzyme e_3 that undergo the following reactions

$$X + X\text{-}Y\text{-}Y \xrightarrow{e_1} 2\ X\text{-}Y$$

$$X\text{-}Y + Z \xrightarrow{e_3} X + Z\text{-}Y$$

which comprises adding the other materials sufficient for the reactions to proceed, and observing the loss of Z or X-Y-Y or the formation of Z-Y.

4. A method according to claim 2, wherein Z-Y is phosphoenolpyruvate or glucose-6-phosphate.

5. A method according to any of claims 2 to 4, wherein Y is a phosphate group.

6. A method according to claim 5, wherein X, X-Y and X-Y-Y are nucleotide phosphates.

7. A method according to claim 6, wherein X, X-Y and X-Y-Y are AMP, ADP and ATP, respectively.

8. A method according to any preceding claim, for detecting ADP and/or ATP.

9. A method according to claim 8, for detecting micro-organisms.

10. A method according to any preceding claim, which comprises observing the formation of P.

11. A method according to claim 10, which is conducted in the presence of one or more substances that generate a signal on the formation of P.

12. A method according to claim 10, which comprises isolating P, and determining its presence by the addition of one or more substances as defined in claim 11.

13. A method according to claim 11 or claim 12, wherein the signal is a visible colour.

14. A kit of reagents suitable for use in a method according to any of claims 11 to 13, the reagents comprising the said other materials and the said one or more substances, in one or more containers.

FIG. 1

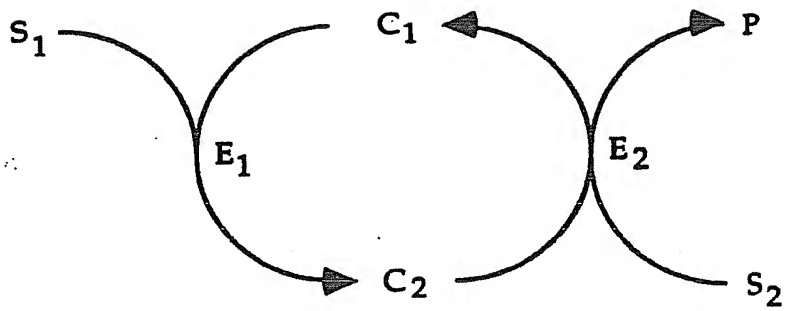


FIG. 2

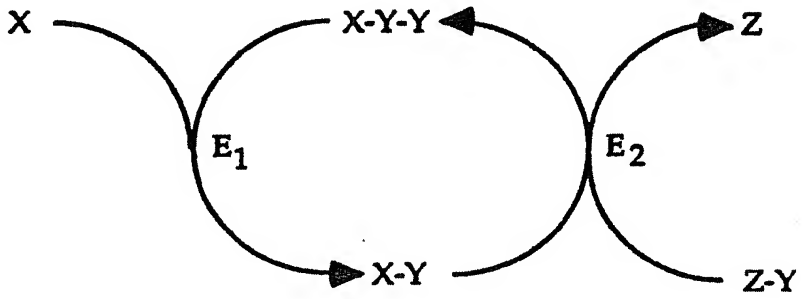


FIG. 3

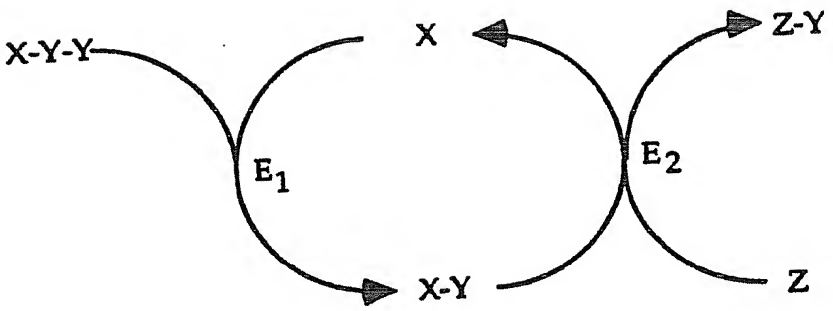


FIG. 4

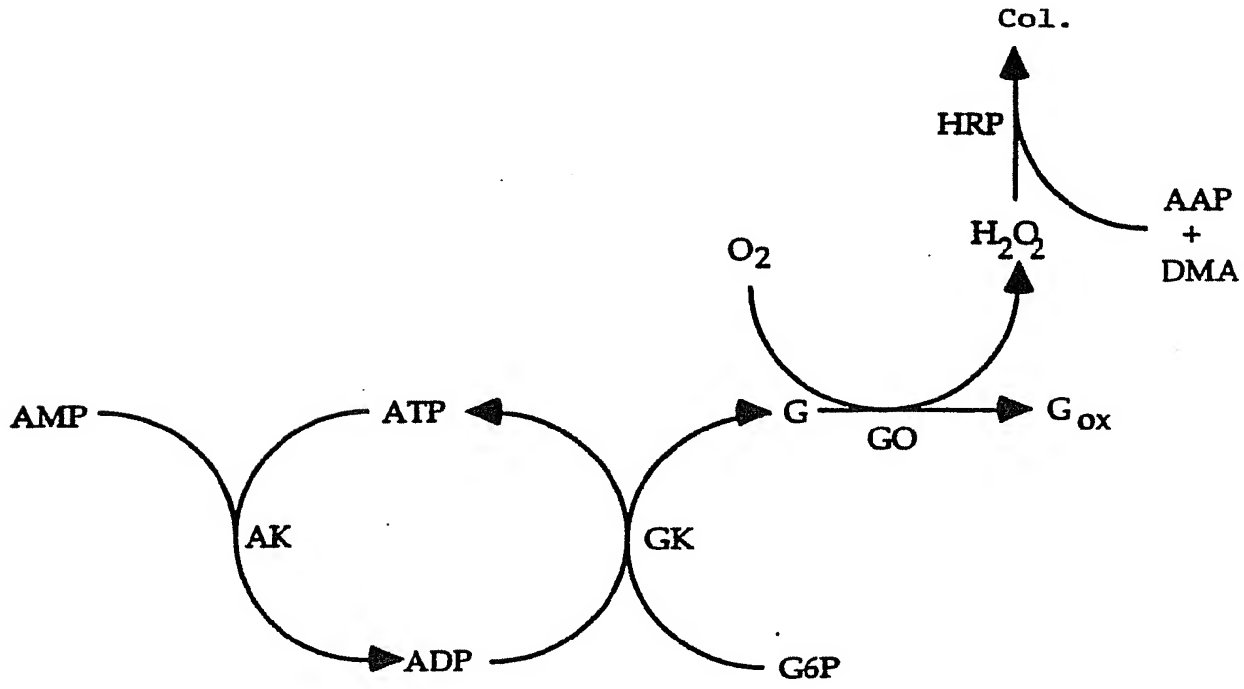
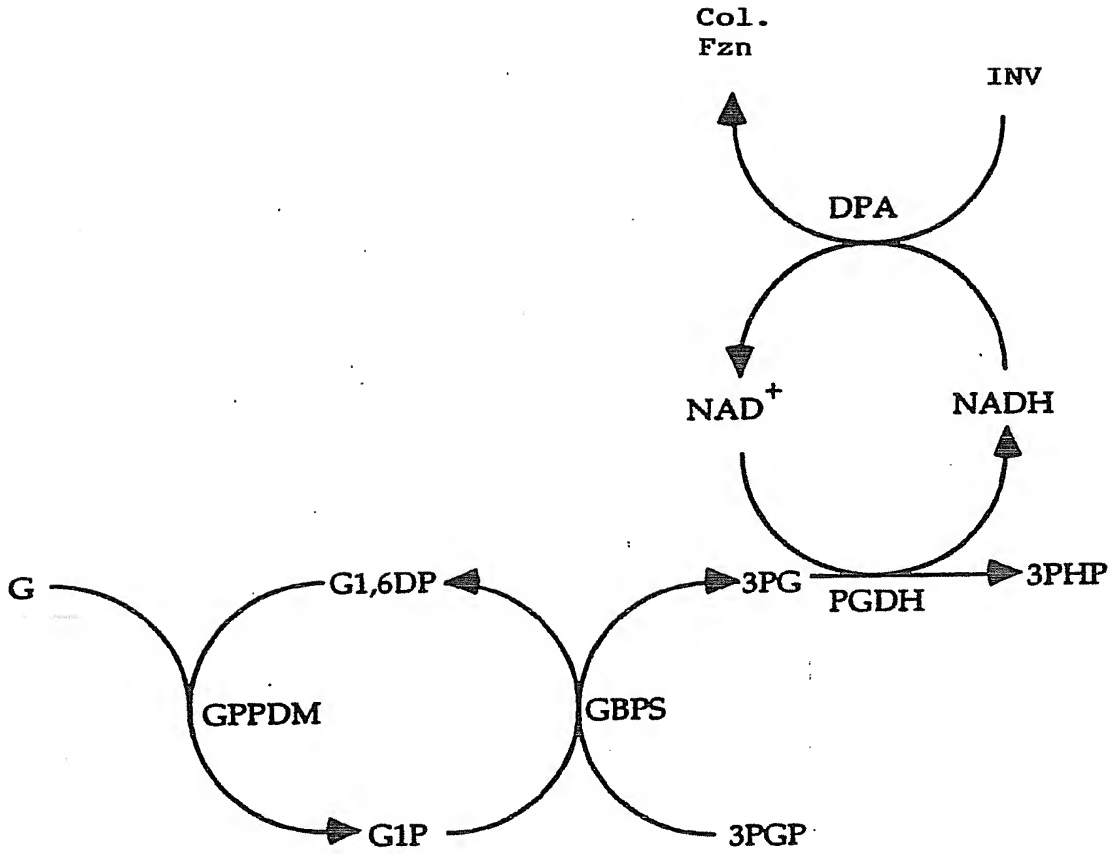


FIG. 5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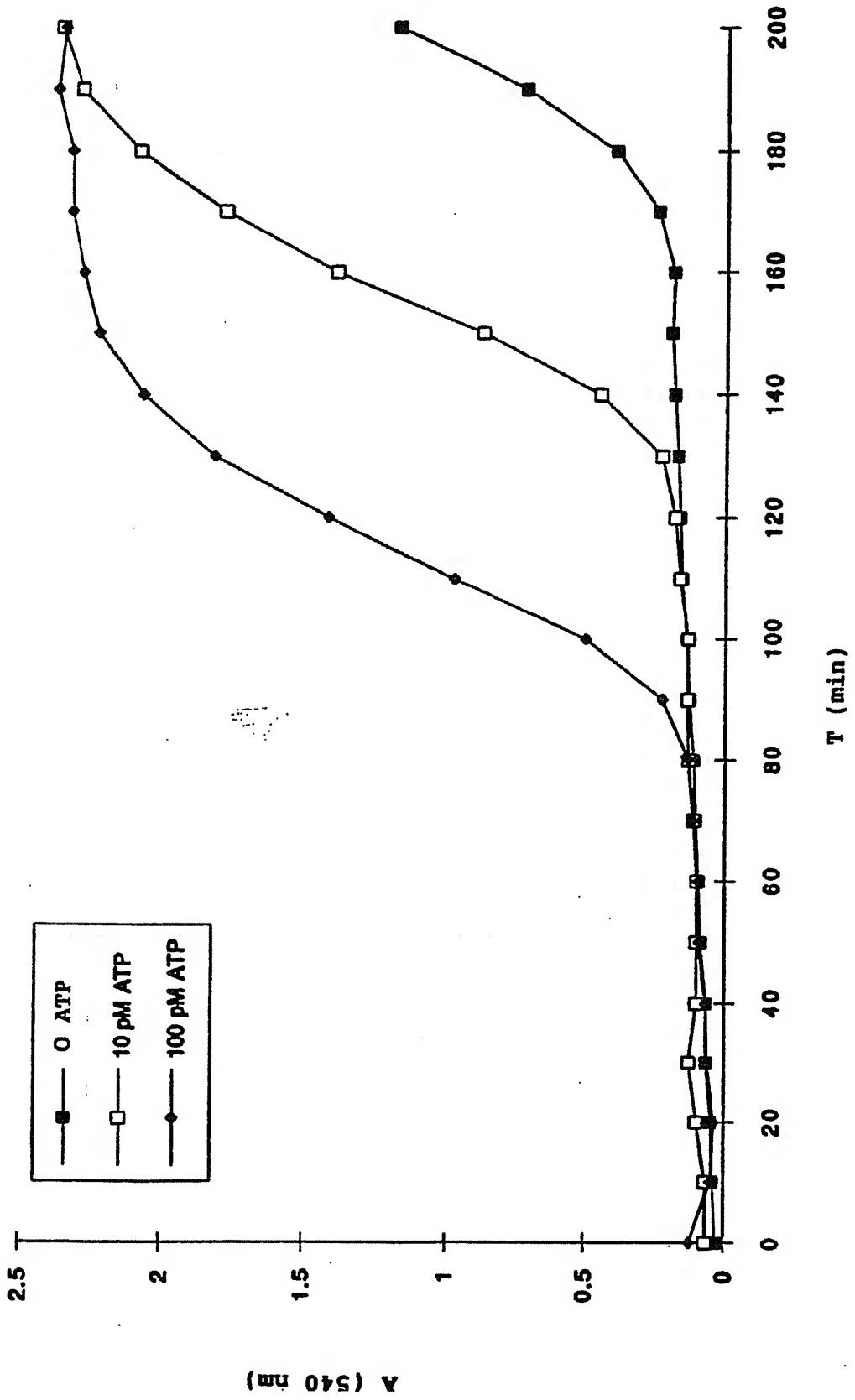

Fig6.

INTERNATIONAL SEARCH REPORT

Inten ..al Application No
PCT/GB 94/00783

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12Q1/00 C12Q1/48 C12Q1/54

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 327 952 (MILES INC.) 16 August 1989 see page 10, line 15 - line 36 ---	1-14
X	PATENT ABSTRACTS OF JAPAN vol. 13, no. 205 (C-595) 15 May 1989 & JP,A,01 023 900 (SANKYO CO LTD) 26 January 1989 see abstract ---	1-14
X	BIOCHEMICAL SOCIETY TRANSACTIONS, vol.19, no.160S, April 1991, LONDON R. S. CHITTOCK ET AL. 'LIGHT AMPLIFICATION BY A COUPLED BIOLOGICAL SYSTEM: ATP, FIREFLY LUCIFERASE AND RECYCLING OF ATP.' cited in the application see the whole document --- -/--	1-14

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
11 July 1994	26.07.94

Name and mailing address of the ISA	Authorized officer
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 193 895 (ABBOTT- LABORATORIES) 10 September 1986 see page 19, line 10 - line 14 ---	1-14
A	EP,A,0 102 504 (MILES LABORATORIES INC.) 14 March 1984 ---	
A	GB,A,2 055 200 (S. KOLEHMAINAN ET AL.) 25 February 1981 cited in the application ---	
A	EP,A,0 060 123 (C. H. SELF ET AL.) 15 September 1982 cited in the application -----	

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter ..al Application No
PCT/GB 94/00783

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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		JP-A-	2031697	01-02-90
		US-A-	5200325	06-04-93
EP-A-0193895	10-09-86	US-A-	4743561	10-05-88
		CA-A-	1263073	21-11-89
		JP-A-	61212762	20-09-86
EP-A-0102504	14-03-84	US-A-	4460684	17-07-84
		AU-B-	540076	01-11-84
		AU-A-	1403883	09-02-84
		CA-A-	1185154	09-04-85
		JP-C-	1718441	14-12-92
		JP-B-	4007198	10-02-92
		JP-A-	59048099	19-03-84
GB-A-2055200	25-02-81	NONE		
EP-A-0060123	15-09-82	US-A-	4446231	01-05-84
		US-A-	4595655	17-06-86